Pseudomonas aeruginosa Alginate in Cystic Fibrosis Sputum and the Inflammatory Response

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Alginate, a viscous polysaccharide from mucoid Pseudomonas aeruginosa, may interfere with the host defenses in patients with cystic fibrosis and chronic P. aeruginosa lung infection. The alginate concentration in the sol phase of expectorated sputum was quantitated by a biochemical method and a newly developed enzyme-linked immunosorbent assay. There was a high degree of correlation between the methods, and the concentration of alginate ranged from 4 to 101 μg/ml with a median of 35.5 μg/ml when measured by enzyme-linked immunosorbent assay. Alginate could not be detected in the bronchial secretions from patients without P. aeruginosa infection. In vitro investigation of alginate did not show any activation of the alternative pathway of complement, as determined by a hemolytic kinetic assay and a test for neutrophil chemotaxis. At a high concentration, P. aeruginosa alginate caused a slight activation of the classical pathway of complement. Alginate did not cause neutrophil chemotaxis by itself but was able to reduce the neutrophil chemotactic response to N-formylmethionineleucylphenylalanine and forzymosan-activated serum. P. aeruginosa and seaweed alginate were able to prime neutrophils for increased N-formylmethionineleucylphenylalanine-induced neutrophil oxidative burst, as determined by chemiluminescence. Because of its ability to prevent attraction of neutrophils to the site of infection, lack of complement activation, and ability to enhance neutrophil oxidative burst, alginate from P. aeruginosa may contribute to the persistence and pathogenesis of chronic P. aeruginosa infection in cystic fibrosis.

Mucoid Pseudomonas aeruginosa is characteristically associated with chronic lung infection in patients with cystic fibrosis (CF) (5), where up to 80% of the patients may harbor mucoid strains. Mucoid P. aeruginosa infection is associated with a pronounced antibody response and a poor prognosis (15), whereas infection with nonmucoid strains seems to be less virulent in CF patients (11). Presumably, there exists a selective survival advantage which favors the chronic persistence of mucoid strains despite a vigorous immune response (29) and normal phagocytic cell function (41) in CF patients. The mucoid substance, a hydrophilic polyanionic polysaccharide, alginate, is therefore considered one of the virulence factors produced by P. aeruginosa (13).

Once established in the lungs, P. aeruginosa exists in the form of mucoid microcolonies (21), and it is rarely eliminated despite an abundance of phagocytic cells in bronchial secretions, mainly represented by polymorphonuclear leukocytes (PMNs) (43). It has been shown that mucoid strains are phagocytosed less readily than nonmucoid strains (1, 8). After treatment with alginate, the phagocytosis of mucoid strains by human monocytes was similar to that of nonmucoid strains (6). By using a crude alginate extract, Schwartzman and Boring (38) found that phagocytosis by rabbit PMNs of one mucoid strain is inhibited, whereas no effect is seen when testing phagocytosis of a nonmucoid strain. Mucoid strains are not phagocytosed by rabbit alveolar macrophages (32), and an inhibitory effect of alginate on the uptake of P. aeruginosa by monocytes (27, 39) has been demonstrated, which was suggested to be caused by the viscous nature of alginate (39).

The clinical correlation of the above findings is not entirely clear, since the concentration of alginate in the lungs is unknown. In this study, we have estimated the concentration of alginate in sol phase of bronchial secretions and used purified alginate preparations to further investigate the interaction of P. aeruginosa alginate with complement and PMNs in vitro.

MATERIALS AND METHODS

Sputum samples. Bronchial secretions were collected by expectoration for 30 to 60 min from 22 patients with cystic fibrosis and chronic P. aeruginosa lung infection and ultracentrifuged for 4 h at 105,000 × g at 4°C according to a previously described procedure (36). The sol phase samples were stored at −20°C until analysis. Sputa from nine patients with chronic lung disease but without P. aeruginosa infection were treated in a similar way and used as controls.

Purified P. aeruginosa alginate. Alginate was purified from two isolates of mucoid P. aeruginosa obtained from bronchial secretions from two patients with CF (strains 8734 and 9721 [28]). Briefly, the organisms were grown on a solid medium for 72 h at 35°C; the mucoid material was scraped off, centrifuged, heated to 80°C for 30 min, precipitated twice with ethanol to a final concentration of 80% (vol/vol), washed repeatedly with ethanol, dissolved in phosphate-buffered saline, digested with nucleases, precipitated with ethanol and fractionated by ion exchange chromatography on a DEAE-Sephadel (Pharmacia, Uppsala, Sweden) column with an ammonium carbonate gradient from 0.05 to 1 M. Fractions containing uronic acid in the carbazole-borate assay (20) were pooled, dialyzed, and freeze-dried. The alginate content was 100% when compared with a standard of seaweed alginate. Previously, the alginate preparations have been shown to contain only trace amounts of protein.

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and lipopolysaccharide (LPS) that had no immunological reactivity (28).

**Seaweed alginate.** Commercial seaweed alginate was obtained from Grindsted Products, Brabrand, Denmark.

**Alginate concentration in sputum.** (i) Carbazole assay. Sol phase samples (0.5 ml) were heated to 80°C for 15 min and centrifuged, and the supernatant was dialyzed against 0.9% saline. Cold ethanol was added slowly to a final concentration of 80% (v/vol), and various amounts of a whitish precipitate were formed. After 1 h at 4°C, the mixture was centrifuged, the supernatant was discarded, and the pellet was dissolved in 0.5 ml of isotonic saline. Uronic acid content was analyzed by the carbazole-borate method (20) with mannuronolactone (Sigma Chemical Co., St. Louis, Mo.) as an internal standard, and the A\(_{350}\) was read.

(ii) ELISA. A competitive enzyme-linked immunosorbent assay (ELISA) was developed to measure the concentration of alginate in secretions. Rabbits were immunized for 3 months with a mixture of three immunologically cross-reactive *P. aeruginosa* alginate preparations (28), and immunoglobulin G and immunoglobulin A were purified by precipitation with ammonium sulfate and ion-exchange chromatography (10). To appropriate dilutions of the mono-specific, polyclonal rabbit immunoglobulin to alginate, 40 \(\mu\)l of heat-treated sputum sol phase secretion was added, and the mixture was incubated for 1 h at 37°C and then overnight at 4°C to allow absorption of alginate, if present, in sol phase samples. Purified *P. aeruginosa* alginate, in concentrations of 3.1 to 200 \(\mu\)g/ml in saline, was used as an internal standard. Microtiter plates (96-well; Mikrowell, Nunc, Roskilde, Denmark) were coated with *P. aeruginosa* alginates in phosphate-buffered saline (pH 7.4). Absorbed rabbit antialginate immunoglobulin (100 \(\mu\)l) was added to the wells and allowed to react for 1 h at 37°C. After being washed with phosphate-buffered saline–Tween 20, peroxidase-conjugated swine anti-rabbit immunoglobulin G (Dakopatts, Glostrup, Denmark) was added and the reaction was visualized with phenylenediamine and \(\text{H}_2\text{O}_2\) (28). The mean optical density of triplicate determinations was used for calculation of the concentration of alginate in sol phase samples. The correlation coefficients for the standards were greater than 0.95.

**Calculation of the alginate concentration in mucoid microcolonies.** Experimentally, it was attempted to estimate the concentration of alginate in mucoid microcolonies by measuring the alginate concentration in colonies on a blood agar plate. It was assumed that these colonies, grown on a plentiful nutrient medium, would resemble those colonies growing in vivo in the lung. Mucoid *P. aeruginosa* was plated out, and the diameters of 10 colonies were measured. The colonies were scraped off and suspended in isotonic saline, and the concentration of alginate was measured by the carbazole-borate assay. The shape of a colony was assumed to be a segment of a sphere not exceeding the size of a hemisphere. The volume of a colony was calculated accordingly, and the concentration of alginate per colony was estimated.

**Complement activation.** The effect of alginate on the hemolytic activity of normal human serum was estimated by its effect on the hemolysis rate in a kinetic assay of the alternative and classical complement pathways (26). The time needed to accomplish 50% lysis of rabbit erythrocytes (alternative pathway) or antibody-coated sheep erythrocytes (classical pathway) was measured with and without preincubation with alginate in a final concentration of 240 and 1,000 \(\mu\)g/ml with fresh normal human serum. Preincubation was carried out at room temperature for 30 min. Buffer served as a control. If alginate reduced the activity of complement in serum, then lysis half time would increase as compared with the buffer control.

**Isolation of PMNs.** Citrated peripheral blood from healthy volunteers was sedimented with dextran and sodium metrizoate-Ficoll (Lymphoprep, Nyegaard, Oslo, Norway), which separates PMNs (3). Remaining erythrocytes were removed by hypotonic lysis. The neutrophils were suspended in Gey's balanced salt solution (GBSS; pH 7.2) with 1% human serum albumin. More than 98% of the cells were PMNs, and cell viability, assessed by trypan blue exclusion, was greater than 97%.

**PMN chemotaxis.** The chemotactic assay was performed in a modified Boyden chamber assay by using 3-\(\mu\)m-pore-size membrane filters (Millipore Corp.) (16). Alginate was dissolved in GBSS and mixed with either fresh human serum or buffer to a final concentration of 1,000 \(\mu\)g/ml, incubated for 30 min at 37°C, and placed in the lower chamber. PMNs (2 \(\times\) 10\(^6\)) were used on the upper side of the chamber. After 2.5 h of incubation at 37°C, the filters were removed, fixed in absolute ethanol, and stained with hematoxylin. Five random fields were counted for each PMN concentration of PMNs per high-power field attracted by zymosan-activated human serum (ZAS) served as the positive control. The ability of alginate to inhibit the PMN chemotaxis was tested by incubating two preparations of alginate in concentrations ranging from 1 to 1,000 \(\mu\)g/ml with 2 \(\times\) 10\(^5\) PMNs (in GBSS with 2% human serum albumin) for 30 min at 37°C. The chemotactic activity of cells preincubated with alginate towards either ZAS or N-formylmethionylleucylphenylalanine (FMLP) was determined.

**Neutrophil chemiluminescence.** A luminol-enhanced chemiluminescence assay, described previously, was used (17). Equal volumes of various concentrations of each alginate preparation were preincubated with 2 \(\times\) 10\(^5\) PMNs per ml for 30 min at 37°C. One milliliter of the cell suspension was then added, either after being washed with GBSS several times or without being washed, to glass scintillation vials containing 4 ml of Krebs Ringer buffer, 0.5 ml of 10\(^{-5}\) \(\text{M}\) FMLP, and 50 \(\mu\)l of luminol (5-amino-2,3-dihydro-1,4-pthalazinedione; Sigma Chemical Co., St. Louis, Mo.). Sequential 0.5-min counts were taken on each vial over a period of 90 min in a Beckman L-8000 scintillation counter.

**Statistical analysis.** Nonparametrical methods were used: the rank sum test for comparison of distributions between groups and the Spearman rank correlation test for correlation analysis. The level of significance used was 5% (two-tailed).

### RESULTS

**Alginate concentration in sputum.** The carbazole-borate assay reacted with sputum sol phase samples, yielding a color that was slightly different from the pure purple color of the mannuronolactone standard and that seemed to be caused by impurities. The absorbance spectrum showed two peaks, at 410 and 530 nm, whereas the major peak of mannuronolactone was at 530 nm. The contribution of unspecific reaction was minimal at 530 nm (data not shown). There was a significant correlation between the chemical and immunological methods for determining the concentration of alginate in sputum (Fig. 1; Spearman rho, 0.77). By using the chemical method, the median concentration of alginate in CF sputum sol phase samples was 37 \(\mu\)g/ml (range, 1 to 94 \(\mu\)g/ml), and with the inhibition ELISA, the median was 35.5 \(\mu\)g/ml (range, 4 to 101 \(\mu\)g/ml). This was
significantly higher than the concentration of alginate in control sol phase samples, in which alginate could not be detected (Fig. 2).

The volume of a microcolony was calculated to be between 1.16 to 9.5 μl, assuming that the volume of the segment ranged from one-fifth to one-half of the volume of a sphere. The corresponding concentration of alginate in a colony ranged from approximately 3 to 0.4 mg/ml.

**Interaction of alginate with complement.** Neither of the two tested purified alginates were able to activate the classical or the alternative pathway of the complement cascade at a concentration of 240 μg/ml, but a slight activation of the classical pathway was found at 1,000 μg/ml. The degree of activation was modest, albeit exceeding the analytical variation of the assay, and it corresponded to a reduction of serum hemolytic activity of 7%.

**Effect of alginate on PMN chemotaxis.** The two alginate preparations were not able to attract PMNs at a concentration of 1 mg/ml, irrespective of whether they were mixed with buffer or fresh human serum. The response was less than 2% of that of the positive control of ZAS and comparable with that of buffer alone. Table 1 shows that alginates in a dose-dependent manner and in concentrations that may be obtained in vivo were able to reduce the neutrophil chemotactic activity of ZAS and FMLP. The reduction in activity was more pronounced with FMLP as a chemoattractant than with ZAS.

**Effect of alginate on PMN chemiluminescence.** Alginate by itself, up to a concentration of 1,000 μg/ml, did not induce any chemiluminescence response in PMNs. Table 2 shows that alginates enhance the FMLP-induced neutrophil chemiluminescence and that this enhancement is dose dependent. Also, seaweed alginate was able to prime neutrophils for increased chemiluminescence. The ability to prime the neutrophils for enhanced chemiluminescence was maintained even after the cells that were preincubated with alginate were washed extensively with GBSS.

**DISCUSSION**

The mucoid, extracellular polysaccharide of *P. aeruginosa*, alginate, has been shown to be antiphagocytic and to scavenge free radicals released from neutrophils (22, 40). The inhibitory activity of alginate on host phagocytic cell function may give alginate-producing, mucoid strains of *P. aeruginosa* a survival advantage in the lungs of patients with CF. This may explain the chronicity of the infection. Also, it has been suggested that alginate, by its physicochemical properties, may contribute to the increased viscosity of airway secretions that is characteristic for patients with CF (33).

All of these observations are, however, based on in vitro experiments, and the concentrations used have been arbitrary because alginate has never been quantitated in lung samples.

**TABLE 1.** The inhibitory effect of *P. aeruginosa* alginate on neutrophil chemotaxis towards two chemoattractants

<table>
<thead>
<tr>
<th>Chemotaxis</th>
<th>Alginates from strain 8754</th>
<th>Alginates from strain 9721</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alginate concn (μg/ml)</td>
<td>ZAS</td>
<td>FMLP</td>
</tr>
<tr>
<td>1,000</td>
<td>51 ± 13</td>
<td>39 ± 10</td>
</tr>
<tr>
<td>500</td>
<td>94 ± 9</td>
<td>67 ± 8</td>
</tr>
<tr>
<td>250</td>
<td>83 ± 6</td>
<td>73 ± 4</td>
</tr>
<tr>
<td>100</td>
<td>ND*</td>
<td>92 ± 3</td>
</tr>
</tbody>
</table>

* Chemotaxis is expressed as a percentage of the control and are the mean ± standard error of four experiments.

* ND: Not done.
secretions. Including the results from the present study, three different laboratories have demonstrated the presence of alginate in CF sputum by using diverse methods such as morphological, immunological, and chemical methods. By using light microscopy, Atkinson et al. (12) showed P. aeruginosa to be mucoid in vivo in CF lungs. By electron microscopy, the bacteria reside in vivo encased in an extensive polysaccharide matrix of mucoid microcolonies (21). With an immunofluorescence technique, two CF lung autopsy specimens reacted with a monoclonal antibody specific for P. aeruginosa alginate (42). Our study showed that alginate was detectable by ELISA and by the carbazole-borate assay of expectorated sputum from patients with CF and chronic P. aeruginosa infection and could be quantitated. These direct observations are furthermore confirmed by finding antialginate antibodies in serum from patients with CF (4, 28, 31), which provides evidence for in vivo production of alginate in the CF lung. Only one other report exists in which alginate has been quantitated in biological fluids; a patient with meningitis with a mucoid strain of P. aeruginosa had a uronic acid concentration in the cerebrospinal fluid of 40 µg/ml, measured by the carbazole-borate method (44).

By using the same biochemical methods, we showed in this study that the sol phase samples of expectorated sputum of CF patients contained alginate in concentrations ranging from 1 to 100 µg/ml, with a median value of approximately 40 µg/ml. These concentrations in the sputum will not, considering the almost normal level of divalent cations in lung secretions (19, 23), cause the alginate to become highly viscous, and we therefore conclude that at these concentrations, alginate probably does not contribute very much to the stickiness of CF sputum.

The carbazole-borate assay is sensitive to impurities (20). The results of the chemical assay were therefore confirmed by developing an inhibition ELISA by using hyperimmune rabbit antialginate immunoglobulins. The correlation between these two independent tests was very high (Fig. 2). The ELISA may therefore be useful for measuring alginate in biological fluids in which contaminants may interfere with the specificity of the chemical method.

The concentration of alginate measured in sputum is representative only of that found in the larger airways, but, because of dilution with host secretions, the figure does not necessarily reflect the highest concentration of alginate that may be found in the lungs. By calculating the alginate content of in vitro colonies, we assume that the concentration of in vivo mucoid microcolonies is at least 10-fold higher than that in the larger airway secretions. In order to investigate the interaction of alginate with the immune cells of the host, a range of alginate concentrations will therefore have to be employed.

Activation of complement provides the host with a way of protecting itself against invading microorganisms, and activation can be triggered by bacterial carbohydrates such as LPS (24) or peptidoglycan (30). We tested the ability of purified P. aeruginosa alginate to activate complement in two different assays. The first assay was a hemolytic kinetic assay in which the ability to form the lytic C5b9 complex was assessed, and the second was a neutrophil chemotactic assay in which C5a-driven chemotaxis was determined. The two alginate preparations tested were unable to activate the alternative pathway in the hemolytic assay, and only at a very high concentration did alginate activate the classical pathway slightly. When mixed with fresh normal serum as the complement source, no neutrophil chemotaxis was observed, confirming the inability of alginate to activate complement. LPS activates the alternative pathway (24), and our results indicate that the alginate preparations were free of biologically active LPS. Sputum sol phase samples from CF patients infected with P. aeruginosa have been shown to contain split products of complement component C3, which was seen as evidence of complement activation occurring in vivo in the lungs (37). It has also been found that mucoid P. aeruginosa can activate complement (34), but, according to our results, this is not likely to be caused by the alginate extracellular matrix. We suggest that alginate, by its inability to activate complement, protects the alginate-producing bacteria from lysis and complement opsonization which may contribute to the persistence of mucoid strains.

Complement activation is an important initial step in the inflammatory reaction, because C5a attracts phagocytic cells to the site of infection. Another chemotactic factor is bacterial oligopeptides such as FMLP. In this study, we have shown that alginate is not a chemotaxant, either on its own or via the complement system, and per se cannot be responsible for the abundance of PMNs present in CF sputum. However, we could show that alginate could inhibit C5a-driven chemotaxis (induced by ZAS) or FMLP-generated chemotaxis in a concentration-dependent manner. This could be because of its ability to chelate divalent cations necessary for optimal human granulocyte chemotaxis (7). Although a different method was used, our results confirm those obtained by Stiver et al. (45), who used an agarose technique to demonstrate that alginate reduced the distance PMNs were able to migrate towards FMLP.

PMNs constitute the majority of phagocytic cells present in CF sputum, and we examined the influence of alginate on the function of neutrophils as measured by chemiluminescence. Mucoid P. aeruginosa has been shown to release significantly more superoxide radical than nonmucoid P. aeruginosa (2). Alginate per se did not have any influence on the PMN response but was able to modulate the response to known activators. When PMNs were preincubated with alginate, the response to FMLP was enhanced by up to threefold that of FMLP alone. A similar finding was observed by Simpson et al. (40) with a concentration of alginate of 50 µg/ml. However, they found, in contrast to our results, that the chemiluminescence response at higher concentrations of alginate was inhibited, which agrees with the results of Learn et al. (22) that alginate is a scavenger of free radicals released by neutrophils. The purity and/or the nature of the alginate used might explain the differences between the results. Consistently, we found a concentration-
dependent enhancement of the PMN response that was present even when preincubated alginic cells were washed. This indicates that alginic induced an altered state of reactivity in the phagocytic cell and that it was not an effect due to an interaction between FMLP and alginic itself. The same reaction was observed with seaweed alginic, which is chemically closely related to bacterial alginic. LPS from gram-negative bacteria has been shown to have a similar enhancing effect (29), and it has previously been demonstrated that the heat-stable fraction of sputum sol phase samples from CF patients also is able to prime neutrophils (18). On the basis of the current data, alginic could, together with LPS, account for this effect.

The progressive damage to pulmonary tissue is probably due to an immune complex-mediated, complement-triggered release of endogenous host proteases and toxic oxygen radicals from neutrophils, as is typical of a type III hypersensitivity reaction (14, 25, 35). In this study, we have examined the interaction of alginic with key elements of the inflammatory system. Alginic did not contribute to the preincubation of PMN to the lungs of CF patients, because it is not a chemoattractant by itself or by activation of complement. On the other hand, it paradoxically inhibited PMN migration to the lungs. However, it may be important in the microenvironment surrounding a mucoid colony to produce a substance that inhibits the movement of phagocytic cells. When the same substance also is unable to activate the innate defense mechanism of the alternative pathway of complement, it may actually provide the bacterium with a means of selecting variants that produce alginic to give it an advantage in terms of phagocytosis and complement-mediated lysis. The presence of alginic may become deleterious to the host, because it stimulates PMN to an enhanced release of toxic oxygen radicals which, in the slow, protracted course of disease, is likely to contribute significantly to the breakdown of lung parenchyma. Alginic was therefore shown to have profound effects on the inflammatory response which define alginic as a defensive and offensive virulence factor in CF lung disease.

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